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1) Crompton et al. Journal of General Virology. 1994; 75 (pt 1): 133-139.

2) Yang et al. Human Gene Therapy. 1998; 9/13: 1929-1937. 9741431

3) Douglas et al. Nature Biotechnology. Nov. 1996; 14 (11): 1574-1578.

4) Hallenbeck et al. (Advances in Experimental Medicine and Biology. 2000; 465: 37-46.

Thank you.
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HUMAN GENE THERAPY 9:1929–1937 (September 1, 1998)
Mary Ann Liebert, Inc.

Development of Novel Cell Surface CD34-Targeted Recombinant Adenoassociated Virus Vectors for Gene Therapy

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ABSTRACT

Recombinant adenoassociated virus (rAAV) type 2 vectors have been used to transduce a wide variety of cell types, including hematopoietic progenitor cells. For *in vivo* gene transfer, it is desirable to have an rAAV vector that specifically transduces selected target cells. As a first step toward generating an rAAV vector capable of targeting delivery *in vivo*, we have engineered a chimeric protein combining the AAV capsid protein and the variable region of a single-chain antibody against human CD34 molecules, a cell surface marker for hematopoietic stem/progenitor cells. Inclusion of the chimeric CD34 single-chain antibody–AAV capsid proteins within an rAAV virion significantly increased the preferential infectivity of rAAV for the CD34$^+$ human myoleukemia cell line KG-1, which is normally refractory to rAAV transduction. Antibodies against the single-chain antibody and the CD34 protein blocked this transduction. This chimeric vector represents a significant improvement in the host range of rAAV and the first step toward specific gene delivery by rAAV vectors to cells of choice, in this case, hematopoietic progenitor cells, for the treatment of human disease.

OVERVIEW SUMMARY

We have constructed rAAV type 2 vectors encoding modified capsid proteins that allow for cell-type specific targeting to cells that express the CD34 protein. Specifically, a fusion protein was constructed, consisting of the N terminus of the AAV virion protein, VP2, and a single-chain antibody directed against CD34. Vector particles packaged in the presence of the VP2–antibody fusion protein are bound specifically to CD34-expressing KG-1 cells. Moreover, while KG-1 cells were resistant to transduction by unmodified rAAV vectors, the modified vector particles were able to transduce these cells. Antibodies against either the CD34 molecule or the single-chain antibody blocked the modified rAAV virion transduction of KG-1 cells. Although improvements in titer of these cell type-specific targetable vectors will be necessary before implementation in *in vivo* gene therapy, this work demonstrates that the tropism of the rAAV particle can be altered by addition of chimeric capsid fusion proteins during viral particle formation.

INTRODUCTION

RECOMBINANT ADENOASSOCIATED VIRUS (rAAV) vectors have been widely used to efficiently transduce a variety of cells including cell types of the hematopoietic system, brain, muscle, liver, and respiratory tract (Flotte *et al.*, 1993; Kaplitt *et al.*, 1994; Zhou *et al.*, 1994; Fisher-Adams *et al.*, 1996; Xiao and Samulski, 1996). There are a number of advantages in using rAAV as a gene therapy vector. For example, rAAV vectors are stable and can be concentrated to high titers. After transduction, rAAV vectors can either integrate into the host chromosome or exist as stable episomal forms, for long-term gene expression (Flotte *et al.*, 1993; Fisher-Adams *et al.*, 1996). Furthermore, unlike retroviral vectors, which can transduce only actively dividing cells, rAAV vectors can efficiently transduce nondividing cells, resulting in long-term *in vivo* expression, particularly in muscle (Flotte *et al.*, 1994; Podsakoff *et al.*, 1994; Xiao and Samulski, 1996). Moreover, because of the lack of any viral coding sequence, *in vivo* applications of rAAV are unlikely to elicit a host cytotoxic T lymphocyte response

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to viral proteins produced by the transduced cells, which results in diminished transgene expression, as is the case for recombinant adenoviral vectors (Yang *et al.*, 1994).

The features described above make rAAV a good candidate vector for both *ex vivo* gene therapy and certain *in vivo* gene therapy applications, i.e., those in which the target cells are readily accessible. However, for *in vivo* systemic delivery of transgenes to treat human diseases, including hematopoietic system disorders, a targetable rAAV vector is clearly needed in order to specifically and efficiently transduce cells of interest. Furthermore, by targeting rAAV vectors to cells that are otherwise devoid of AAV receptors, the tropism of rAAV vectors can also be expanded.

Retroviral vectors (RVVs) and adenoviral vectors (Advs) have been used for tissue- and cell-specific targeting by various means. However, rAAV has so far not been used for this purpose, largely because limited knowledge is available concerning AAV receptor binding and cell entry. Our strategy to achieve targeted delivery of rAAV is to modify the cellular binding specificity of the rAAV virion. The AAV virion is composed of three capsid proteins, VP1, VP2, and VP3, with a stoichiometry of 1:1:10 (Becerra *et al.*, 1988; Trempe and Carter, 1988). They are encoded by the AAV p40 promoter-driven capsid gene. All three proteins share the same open reading frame, each having a unique initiation codon but the same stop codon, such that the size of the polypeptides is progressively shorter from VP1 to VP3. Earlier work has shown that elimination of any of the three capsid proteins leads to failure to produce infectious AAV particles (Muralidhar *et al.*, 1994); therefore, our initial strategy is to add a ligand of interest to the intact proteins to redirect the infection of AAV vector to specific target cells.

In this study, we incorporate the single-chain fragment variable region (sFv) of a monoclonal antibody against the CD34 molecule, a human cell surface marker for hematopoietic progenitor cells (Civin *et al.*, 1984), to the AAV type 2 virion by linking sFv to the capsid gene. The sFv is composed of variable domains of immunoglobulin heavy chain (V_H) and light chain (V_L) bridged by a linker sequence. In most cases, the binding affinity of sFv to its ligand is comparable to the whole antibody molecule (Whitlow and Filpula, 1991). In several studies, the sFv is used to redirect retrovirus vectors to specific target cells (Somia *et al.*, 1995; Chu and Dornburg, 1997). As a starting point for development of a targetable rAAV vector that can be used for *in vivo* gene therapy with optimal efficiency, we demonstrate that the inclusion of sFv-capsid fusion protein in a rAAV vector results in a significant increase in transduction efficiency to $CD34^+$ cells by the modified rAAV vector.

MATERIALS AND METHODS

Cell culture

Human HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. KG-1 cells, a human acute myelogenous leukemia cell line that is positive for CD34 surface molecules (Civin *et al.*, 1984), were purchased from the American Type Culture Collection (ATCC CCL-246; ATCC, Rockville, MD) and cultured in Iscove modified Eagle's medium supplemented with 20% fetal bovine serum. Anti-CD34 hybridoma my-10 (ATCC HB8483) was cultured in RPMI 1640 medium in the presence of 10% fetal bovine serum. All cells were maintained at 37°C in a 5.0% CO_2 atmosphere.

Cloning of sFv from my-10 hybridoma

The sFv was cloned from my-10 cells, which are an anti-human CD34 hybridoma raised against KG-1 cells (Civin *et al.*, 1984). Briefly, total RNA isolated from my-10 hybridoma was converted into cDNA by avian myeloblastosis virus (AMV) reverse transcriptase. Amplification of the heavy chain and light chain genes was carried out with *Taq* polymerase in polymerase chain reactions (PCRs) using a combination of degenerate primers in the Ig-Primer kit provided by Novagen (Madison, WI), according to the manufacturer protocol. The resulting fragments, about 450 base pairs (bp) for both V_H and V_L, were subcloned into a TA cloning vector (Invitrogen, San Diego, CA) and sequenced. Primer pair $MulgV_H5'$-C and $MulgV_H3'$-2, as well as $MulgkV_L5'$-G and $MulgkV_L3'$-1 (see the manufacturer catalog for detailed sequence information), generated DNA fragments including the N-terminal and C-terminal conserved sequence for the V_L and V_H, respectively. The sequences of multiple clones were compared and consensus sequences were utilized for subcloning into pBlueScript vector (Stratagene, La Jolla, CA) carrying the linker sequence encoding $(GGGGS)_3$ sequences to generate the pSFv plasmid (Fig. 1A). During subcloning, an initiation ATG codon was incorporated into the 5′ end of V_H. The resulting sFv protein, V_H-$(GGGGS)_3$-V_L, contains 116 amino acid residues of V_H at the N terminus, followed by a linker sequence and 112 amino acid residues of V_L at the C terminus (Fig. 1A).

Expression and purification of sFv from bacteria

The sFv gene was subcloned into the pTrc/his vector (Invitrogen; see the manufacturer catalog for details) to generate a bacterial expression plasmid, pTrc/sFv, and was produced as a polyhistidine fusion protein (Fig. 1B) (Hochuli *et al.*, 1987). The *Escherichia coli* bacterial culture of pTrc/sFv was grown to OD_{550} 0.5 before it was induced with a 0.1 m*M* final concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 hr at 37°C. One-step purification of histidine-tagged sFv protein was performed using a nickel column (Invitrogen) under denaturing conditions according to the manufacturer protocol. The eluted fractions (10 μl each) were mixed with 10 μl of 2× sample buffer (Ausubel *et al.*, 1987) and boiled for 5 min. The proteins were analyzed on a 4–20% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel (Novex, San Diego, CA), and stained with Coomassie blue. A Western blot analysis was performed using anti-His C-terminal antibody (Invitrogen) and horseradish peroxidase-conjugated goat anti-mouse antibody (Chemicon, Temecula, CA). An enhanced chemiluminescence (ECL) system from Amersham (Arlington Heights, IL) was used to detect protein signals. Renaturation of purified protein was performed by dialyzing the eluted fractions against 10 mM Tris-HCl (pH 7.8) and 0.1% Triton X-100 at 4°C overnight. Any denatured or nonsoluble proteins were removed by centrifugation before use.

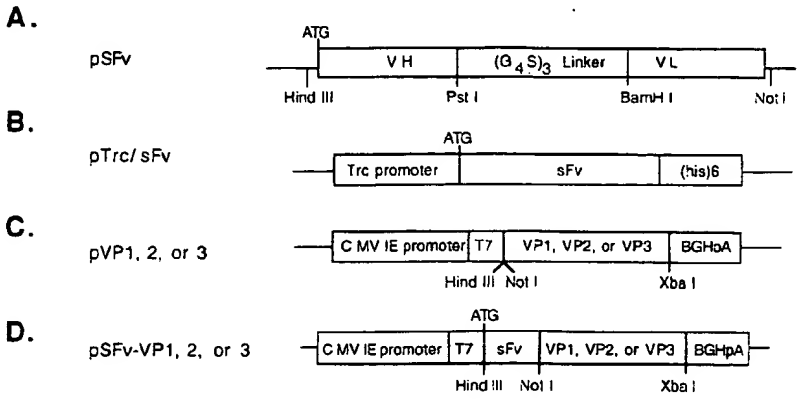

FIG. 1. Plasmid map for pSFv (A), pTrc/sFv (B), pVP1, 2, or 3 (C), and pSFv-VP1, 2, or 3 (D). Some important features and selected restriction sites of the plasmids are outlined. ATG, translation initiation codon; V_H, antibody heavy chain variable region; $(GGGGS)_3$, linker sequence; V_L, antibody light chain variable region; Trc, IPTG-inducible *trpB* and *lacUV5* hybrid promoter; (his)6, histidine-tag for nickel column purification; CMV IE, cytomegalovirus immediate-early promoter; T7, bacterial T7 promoter; BGHpA bovine growth hormone gene poly(A) signal.

Binding of purified sFv to CD34

For the binding of bacterial purified histidine-tagged sFv to CD34 proteins expressed on KG-1 cells, a competitive binding assay was performed. About 1×10^5 KG-1 cells were incubated with or without 1 μg of sFv protein or my-10 monoclonal antibody (MAb) or with the same amount of control bacterial β-galactosidase protein at 4°C (to prevent internalization) for 30 min. About 0.05 μg of phycoerythrin (PE)-conjugated ICH3 anti-CD34 monoclonal antibody (1 mg/ml; Caltag Laboratories, Burlingame, CA) was then added and incubated for an additional 30 min. After two or three washings with phosphate-buffered saline (PBS), the stained cells were subjected to flow cytometry analysis using a FACS Vantage (Becton Dickinson, Mountain View, CA).

Cloning of sFv–AAV capsid fusion proteins

Standard procedures were followed for plasmid construction, growth, and purification throughout the study (Ausubel *et al.*, 1987). Plasmid pAV2 contains a full-length AAV-2 genome, which was purchased from the ATCC (Laughlin *et al.*, 1983). The capsid genes for virion proteins VP1, VP2, and VP3 were amplified by PCR using a 25-bp 3′ primer at the end of the AAV capsid gene open reading frame (ORF) (VP-3′, TGC TCT AGA GCA TTA ACA GAC TTG TAG TTG GAA GT) and 5′ primers corresponding to the start codon of individual capsid genes, i.e., 2203–2229 for VP1 (VP1 2203–2229-Sal, CAC TCG TCG ACA TGG CTG CCG ATG GTT ATC TTC CAG AT), 2617–2640 for VP2 (VP2 5′-2617–2640, CGC CGC TCG AGA TGG CTC CGG GAA AAA AGA GGC CGG T), and 2810–2833 for VP3 (VP3 2810–2833-Xho, CGC CCT CGA GAT GGC TAC AGG CAG TGG CGC ACC AAT). The PCR products were subcloned into a pCDNA3 expression vector (Invitrogen) to generate plasmids pVP1, pVP2, and pVP3, respectively (Fig. 1C). The above-described cloned anti-CD34 sFv gene was placed at the 5′ end of VP1, VP2, and VP3 genes, using *Hin*dIII and *Not*I sites to generate plasmids pSFv-VP1, pSFv-VP2, and pSFv-VP3, respectively (Fig. 1D).

The expression of various AAV virion proteins and their fusion proteins was verified *in vitro* using the TNT (Promega, Madison, WI) transcription and translation kit according to the manufacturer instructions. Briefly, 2 μg of plasmid DNA was added to 50 μl of rabbit erythrocyte lysate in the presence of 10 μCi of ^{35}S-labeling mix (Amersham) and the reaction was allowed to proceed for 1 hr at 37°C. The protein products were subjected to SDS-PAGE and autoradiography.

Production and purification of rAAV vector

For packaging vectors, a previously described gene transfer method (Mamounas *et al.*, 1995) with adenovirus coupled to a DNA–polylysine complex was used for the first batch of vector preparation. Ten million HeLa cells in a T162 flask were "transinfected" with conjugates composed of polylysine-coupled adenovirus (multiplicity of infection, 10) as well as 10 μg of plasmid pAAV/Ad and 10 μg of plasmid pAAVgal. The plasmid pAAVgal is an AAV vector plasmid carrying the bacterial β-galactosidase gene under the control of the cytomegalovirus immediate-early promoter (CMV IE). Plasmid pAAV/Ad provides AAV replication and capsid gene products for packaging of rAAV/gal (Samulski *et al.*, 1989). After 48–72 hr, when about 80% of the cells displayed maximum cytopathic effect, the cells were harvested and resuspended in 1 ml of culture medium. The rAAV was extracted after three cycles of freezing and thawing. The lysates of the cell culture from 30 to 40 T162 flasks were combined and CsCl gradient purification of the rAAV vector was carried out as follows. The saturated CsCl solution was added to the lysates to make the final density 1.4 g/ml. After centrifugation at 35,000 rpm for 18 hr, the fractions containing cell debris and a majority of the adenovirus were removed, and fractions of rAAV were subjected to a second centrifugation step at 35,000 rpm for 48 hr. The resulting CsCl gradient was collected in 1-ml fractions. The fractions with peak concentrations of rAAV virus, based on standard slot-blot analysis of the rAAV genome, were dialyzed against 500 ml of DMEM containing 2% fetal bovine serum (FBS) and 1%

glycerol for 2 hr at 4°C with one change of dialysis buffer. Purified rAAV vectors were stored at −70°C. The residual adenovirus in the rAAV vectors was heat inactivated for 30 min at 56°C before use.

For production of rAAV vectors containing sFv–AAV virion fusion protein, in addition to plasmids pAAV/Ad and pAAV-gal, 10 μg of plasmid pSFv-VP2 was also included separately for each T162 flask in transinfection to generate rAAV vector rAAVsFv-VP2/gal.

Second batches of rAAV/gal and rAAVsFv-VP2/gal were prepared by transfection of A549 cells with Lipofectamine in the presence of adenovirus infection. The resulting rAAV lysates were subjected to HPLC purification (Wampler *et al.*, unpublished data; and Tamayose *et al.*, 1996).

rAAV transduction

For transduction with rAAV vectors, about 2×10^4 KG-1 or HeLa cells were plated in 48-well dishes the day before transduction. To transduce HeLa and KG-1 cells, 100, 10, or 1 μl of rAAV vector stock was added to the cells for 3 hr before fresh medium was added. Two days afterward, the cells were washed and expression of the bacterial β-galactosidase gene was detected by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining by the following procedure: the cells were washed with PBS and fixed in buffer containing 2% formaldehyde and 0.2% glutaraldehyde in PBS for 5 min at room temperature. Finally, the cells were washed with PBS before incubation with staining buffer (5 m*M* potassium ferricyanide, 5 m*M* potassium ferrocyanide, 2 m*M* $MgCl_2$ and X-Gal [1 mg/ml in PBS]) for 4 hr at 37°C. The positive cells were scored by counting blue cells under a microscope. The titer of virus is determined by the dilution at which between 10 and 200 blue cells were counted.

For the antibody blocking assay, about 1×10^4 KG-1 or HeLa cells were plated in 96-well dishes the day before transduction. Before transduction, the HPLC-purified rAAV vectors were concentrated onefold by speed vaccum. One hundred microliters of concentrated virus was incubated with either no antibody, antibody against AAV whole particle (American Research Products, Belmont, MA), antibody against CD34 (my-10 hybridoma supernatant), or antibody against sFv generated from rabbit (HTI, San Diego, CA) for 1 hr at room temperature in a total volume of 200 μl. Five microliters of the mixture was used to transduce HeLa cells while the remaining 195 μl was used to transduce KG-1 cells. Additional PBS was added to the cells to bring the total volume to 200 μl during the transduction process. After transduction for 3 hr at 37°C, the virus was removed and fresh medium was added. Two days after transduction, cells were stained for β-galactosidase gene expression as described above.

Binding of sFv-containing rAAV vectors to CD34 molecules

To analyze the binding of CsCl-purified rAAV/gal vectors and rAAVsFv-VP2/gal vectors to CD34 molecules, about 1×10^5 KG-1 cells were incubated with and without 1×10^{10} rAAV particles of virion–sFv fusion protein containing rAAVsFv-VP2/gal or the same amount of wild-type virion rAAV/gal particles at 4°C for 30 min. About 0.05 μg of PE-conjugated ICH3 antibody was added and incubated for an additional 30 min. After washing with PBS, the stained cells were subject to flow cytometry analysis.

RESULTS

Cloning of sFv from my-10 hybridoma

As described in Materials and Methods, the V_H and V_L genes were amplified by PCR using degenerate primers and assembled so that the C terminus of V_H is linked to the N terminus of V_L by a linker sequence (triplicate of Gly-Gly-Gly-Gly-Ser). Three of six of the heavy chain primer pairs produced PCR products; however, only one of them gave rise to a DNA sequence containing a continuous ORF and encoding the consensus sequence of V_H (i.e., VQLKQ at the N terminus and LTVSS at the C terminus). Similarly, only one PCR product from three light chain primer pairs had a continuous ORF that encodes the consensus sequence for V_L (i.e., DVVMT at the N terminus and KVELK at the C terminus). The calculated molecular mass of sFv with V_H linked to V_L was 29.4 kDa.

The cloned sFv was expressed in bacteria as a histidine-tagged protein. After induction with IPTG for 3 hr, a 35-kDa protein was detected, which was later purified by nickel column chromatography (Fig. 2A, lanes representing fractions 1–5). Fraction 3 was collected for further analysis. After dialysis and renaturation, the protein remained soluble and was analyzed (Fig. 2A, lane entitled *dialyzed #3*). The increase in apparent molecular mass from the predicted 29.4 kDa for sFv to 35 kDa for the fusion protein on SDS-PAGE was due to the inclusion of other sequences on pTrc/his vectors, including multiple cloning sites, histidine tag, and Myc epitope. The authenticity of the fusion protein was verified by Western blot probing with an antihistidine C-terminal antibody (Fig. 2B). The 35-kDa protein was detected in the induced bacterial total protein as well as in eluted fractions 1–5 and dialyzed fraction 3, but not in uninduced bacteria. As a positive control for antihistidine C-terminal antibody, purified histidine-tagged bacterial β-galactosidase protein was included in the blot (lane entitled *LacZ*), which is approximately 105 kDa in size.

Binding of sFv to CD34

To verify the authenticity of the cloned sFv, we first sought to perform direct staining of KG-1 cells with conjugated sFv; however, we found that conjugation of my-10 antibody usually abolished its binding capability to CD34 molecules. We therefore developed a competitive binding assay with another anti-CD34 MAb, ICH3, for which a PE-labeled product is available, based on the fact that my-10 can compete with ICH3 for binding to CD34 proteins (Gaudernack and Egeland, 1995). To this end, CD34-expressing KG-1 cells were preincubated with 1 μg of histidine-tagged sFv, my-10, or control bacterial β-galactosidase (His-tagged) protein (see Materials and Methods) before PE-labeled ICH3 antibody was added to the cells. Without preincubation with sFv the PE-conjugated ICH3 MAb at 50 ng/200 μl can stain 55.6% of 100,000 KG-1 cells (Fig. 3B), whereas the PE-conjugated mouse IgG has no binding affinity for KG-1 cells (Fig. 3A, 0.4% positive). The addition of nonspecific mouse IgG (Fig. 3C) and histidine-tagged β-galactosidase protein (Fig. 3F) did not interfere with the binding of ICH3 to KG-1 cells with 60.8 and 55.0% of the cells staining posi-

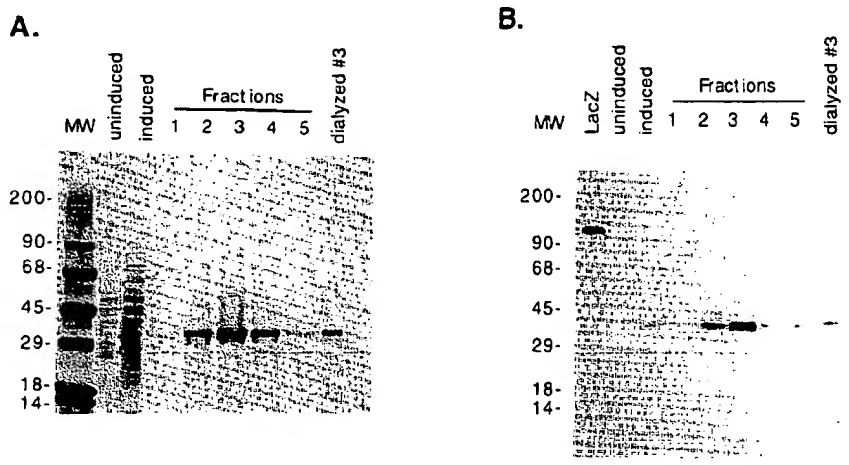

FIG. 2. Expression and characterization of the histidine-tagged sFv in bacteria. **(A)** SDS-PAGE analysis of uninduced and IPTG-induced bacterial total protein and eluted fractions 1–5 from nickel column-purified sFv and dialyzed fraction 3. MW, Molecular mass (kDa). **(B)** Western blot analysis of samples in **(A)** and purified histidine-tagged β-galactosidase protein (lane LacZ) using anti-His C-terminus antibody.

tive, respectively. However, the presence (with preincubation) of 1 μg of purified sFv (Fig. 3E) and my-10 MAb (Fig. 3D) reduced the capacity of ICH3 binding to CD34 by more than 90%, with 5.3 and 1.7% of the cells staining positive, respectively. This demonstrates that the purified sFv can specifically bind to CD34 molecules expressed on the surface of KG-1 cells, possibly with slightly reduced affinity than the parental my-10 MAb.

Expression of sFv–AAV capsid fusion proteins

To incorporate the sFv in the rAAV particles, we subcloned the sFv gene to the N terminus of individual AAV capsid proteins VP1, VP2, and VP3 (Fig. 1D). Subsequently, we tested the expression of the fusion proteins *in vitro*. In TNT (transcription and translation) assays, we detected the AAV VP1, VP2, and VP3 proteins with plasmids pVP1, pVP2, and pVP3 at predicted sizes 90, 72, and 60 kDa, respectively (Fig. 4, lanes 1, 3, and 5, respectively). When plasmids pSFv-VP1, pSFv-VP2, and pSFv-VP3 were added to the TNT reactions, we observed the fusion proteins sFv–VP1, sFv–VP2, and sFv–VP3 at positions corresponding to the deduced molecular masses (122, 106, and 92 kDa, respectively) (Fig. 4, lanes 2, 4, and 6). There were several smaller species of protein products that comigrated with VP1, VP2, or VP3 in lanes 1, 2, 3, 4, and 5. This probably was the result of alternate initiation of translation from either the VP1, VP2, or VP3 start codon.

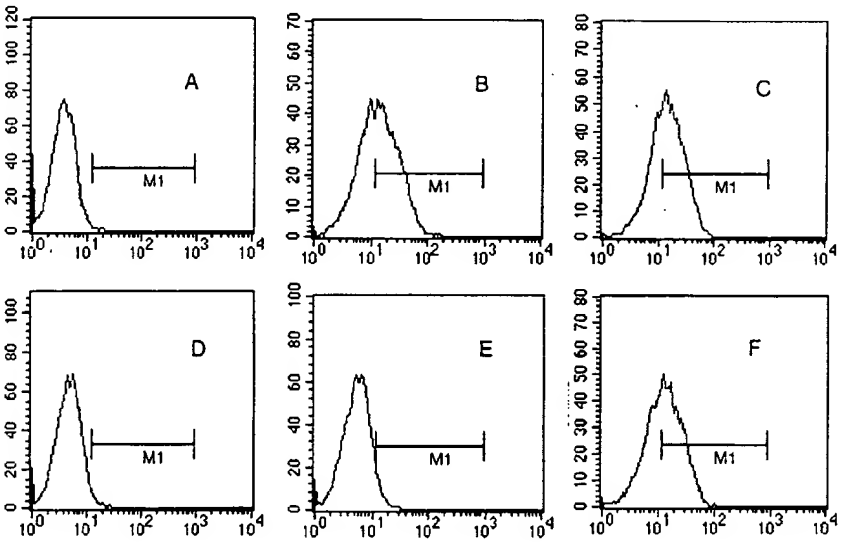

FIG. 3. The purified sFv specifically binds to CD34 molecules. The purified sFv is shown by flow cytometry analysis to block the binding of ICH3 antibody to CD34 on KG-1 cells. KG-1 cells were preincubated with PBS buffer (A and B), mouse IgG_1 (C), purified my-10 (D), sFv (E), or control β-galactosidase protein (F) before incubation with PE-conjugated mouse IgG_1 (A, isotype control) or PE-conjugated ICH3 anti-CD34 MAb (B, C, D, E, and F).

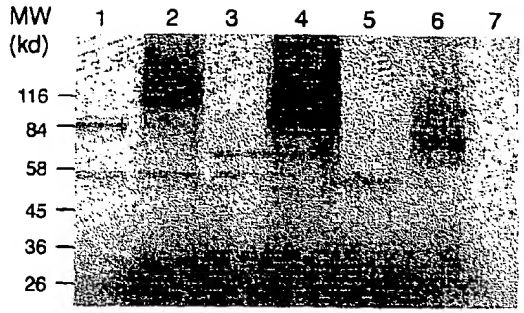

FIG. 4. *In vitro* transcription and translation of AAV virion proteins (VPs) and sFv–VP fusion proteins. The TNT reactions contain 2 μg of plasmid pVP1 (lane 1), pSFv-VP1 (lane 2), pVP2 (lane 3), pSFv-VP2 (lane 4), pVP3 (lane 5), pSFv-VP3 (lane 6), or no plasmid DNA (lane 7). The protein product are subjected to SDS-PAGE analysis and autoradiography. Molecular weights (MW) are shown on the left.

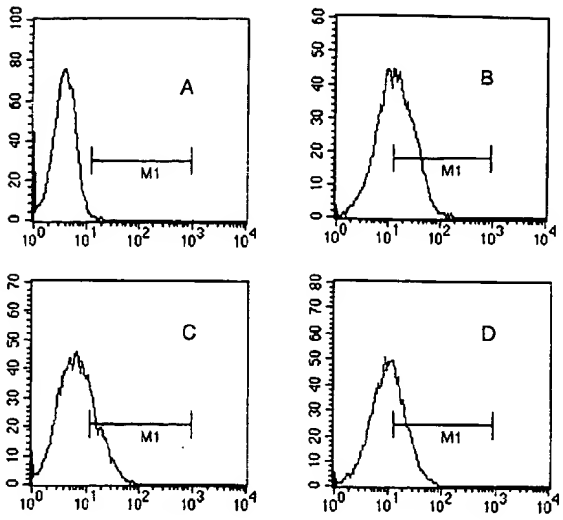

FIG. 5. rAAVsFv-VP2/gal specifically binds to CD34 molecules. The sFv on the surface of the rAAVsFv-VP2/gal vectors is shown by flow cytometry analysis to block the binding of ICH3 antibody to CD34 on KG-1 cells. KG-1 cells were preincubated with PBS buffer (**A** and **B**), CsCl-purified rAAVsFv-VP2/gal vectors (**C**), or wild-type rAAV/gal (**D**) before incubation with PE-conjugated mouse IgG_1 (**A**, isotype control) or PE-conjugated ICH3 anti-CD34 MAb (**B**, **C**, and **D**).

Increased transduction of CD34$^+$ KG-1 cells by sFv-containing rAAV

Initially, we attempted to use either all three sFv–VP fusion proteins or one sFv–VP fusion protein with two other unmodified virion proteins along with an AAV *rep* gene-expressing plasmid to produce rAAV vector with a β-Gal reporter gene. None of the combinations we tested produced detectable rAAV particles. Thus, we decided to include all three wild-type AAV capsid proteins into the packaging process with the addition of a single sFv fusion protein of VP-1, -2, or -3. This produced intact rAAV particles, as confirmed by the standard particle slot-blot assay (data not shown). This method of preparation of modified rAAVsFv-VP2/gal most likely contains a mixture of chimeric and wild-type rAAV virions. Initial screening of these vectors for their infectivity on HeLa cells showed that while the inclusion of any one fusion protein produced infectious rAAV particles, only sFv–VP2 clearly increased the infectivity of rAAV on KG-1 cells, which were resistant to infection by wild-type virion protein rAAV/gal under the conditions tested. The addition of sFv–VP1 or sFv–VP3 had no effect on rAAV infectivity on KG-1 cells (data not shown). We then proceeded to produce large quantities of normal (rAAV/gal) and chimeric rAAV vectors (rAAVsFv-VP2/gal) and purified them by either CsCl gradient or HPLC (Materials and Methods). Table 1 summarizes the infectivity of normal and targeted rAAV vectors on HeLa and CD34$^+$ KG-1 cells. Although comparable transduction efficiencies were observed on HeLa cells by rAAVsFv-VP2/gal and rAAV/gal (see also Fig. 6A and C, respectively), the modified rAAV virion carrying sFv–VP2 fusion protein can transduce KG-1 cells (also Fig. 6D) while the wild-type vector rAAV/gal has no detectable infectivity under the condition tested (also Fig. 6B). This increase in transduction efficiency on KG-1 cells was observed with both CsCl gradient- and HPLC-purified rAAVsFv-VP2/gal vector. The best titer we

TABLE 1. INFECTIVITY OF rAAVsFv-VP2/gal AND rAAV/gal ON HeLa AND KG-1 CELLS

Vector preparation	*Vectors*	*Titer on HeLa (FFU/ml)*[a]	*Titer on KG-1 (FFU/ml)*[a]
CsCl purified	rAAV/gal	1,300,000	<10
	rAAVsFv-VP2/gal	880,000	90
HPLC purified	rAAV/gal	168,000	<10
	rAAVsFv-VP2/gal	46,000	190

[a]FFU/ml (focus formation units per milliliter), is defined as galactosidase transducing units per milliliter of vector preparation. The limit of detection is 10 FFU/ml for both KG-1 and HeLa cells.

TABLE 2. INFECTIVITY OF rAAVsFv-VP2/gal AND rAAV/gal IN THE PRESENCE OF ANTIBODIES

Vector	*Antibodies*	*Titer on HeLa (FFU/ml)*[a]	*Titer on KG-1 (FFU/ml)*[a]
rAAV/gal	No Ab	86,000	<5
	Ab to AAV particle	<200	<5
	Nonspecific IgG_1	95,200	<5
	Ab to sFv	82,400	<5
	Ab to CD34	79,600	<5
rAAVsFv-VP2/gal	No Ab	63,200	430
	Ab to AAV particle	<200	310
	Nonspecific IgG_1	61,400	375
	Ab to sFv	67,200	<5
	Ab to CD34	81,200	60

[a]FFU/ml (focus formation units per milliliter), is defined as galactosidase transducing units per milliliter of vector preparation. The limit of detection is 200 FFU/ml for HeLa cells and 5 FFU/ml for KG-1 cells.

have observed so far is 1.9×10^2 transducing units/ml on KG-1 cells with rAAVsFv-VP2/gal, which, although relatively low, is remarkably better than that of wild-type rAAV.

Specific binding of rAAVsFv-VP2/gal to CD34 molecules

To study the specificity of rAAVsFv-VP2/gal transduction in KG-1 cells, competitive binding assays were performed to investigate whether the CsCl-purified rAAVsFv-VP2/gal blocks PE–ICH3 antibody from binding to CD34 molecules on KG-1 cells (Fig. 5). The PE-conjugated ICH3 MAb at 50 ng/200 μl binds to 55.6% of 100,000 KG-1 cells (Fig. 5B), while the nonspecific PE-conjugated mouse isotype IgG has no binding affinity for KG-1 cells (Fig. 5A, 0.4% positive). Preincubation of 1×10^{10} particles of sFv-VP2 containing rAAV significantly reduced the binding of ICH3 antibody to CD34 molecules from 55.6 to 23.2% (Fig. 5C). In contrast, the same amount of wild-type rAAV/gal reduced the binding of ICH3 antibody to CD34 only from 55.6 to 40.6% (Fig. 5D), presumably owing to nonspecific binding. When the amount of PE-conjugated ICH3 was increased to 200 ng/200 μl, the inhibition by rAAVsFv-VP2/gal was reserved and no longer observed (data not shown). This suggests that the sFv–VP2 fusion protein is located on the surface of rAAVsFv-VP2/gal and that it can mediate the binding of rAAVsFv-VP2/gal to CD34 molecules on the KG-1 cell surface, resulting in specific delivery of AAV vectors into KG-1 cells.

Transduction of KG-1 cells by rAAVsFv-VP2/gal is mediated by interaction of sFv and CD34 molecules

To investigate whether the preferential transduction of KG-1 cells by chimeric rAAV vector was mediated through the interaction of sFv on the surface of rAAVsFv-VP2/gal and CD34 molecules on the surface of KG-1 cells, we performed a series of antibody blocking studies, summarized in Table 2. The HPLC-purified rAAV vectors were first concentrated onefold. They were then preincubated with no antibody or antibody against AAV intact particle, CD34, or sFv before transduction on HeLa and KG-1 cells. Figure 6A and B shows the infectivity of HPLC-purified wild-type rAAV/gal on HeLa and KG-1

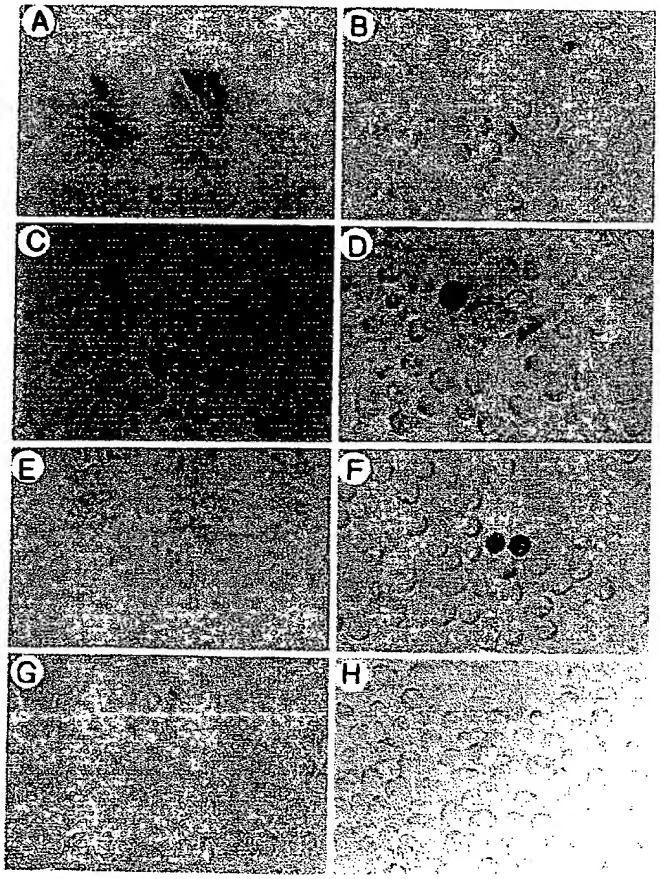

FIG. 6. Transduction of HeLa and KG-1 cells by rAAV vectors. The HPLC-purified rAAV/gal (A and B) or rAAVsFv-VP2/gal (C to H) was preincubated with no antibody (A to D) or antibody against whole AAV particle (E and F), antibody against CD34 (G), or antibody against sFv (H) before they were used to transduce HeLa cells (A, C, and E) and KG-1 cells (B, D, F, G, and H). The transduced cells were stained for the expression of bacterial galactosidase gene by X-Gal staining. Original magnification: (A, C, E) ×100; (B, D, F–H) ×400.

cells, respectively. No detectable infectivity by wild-type rAAV was seen on KG-1 cells (see also Table 2). Figure 6C and D indicates that chimeric rAAVsFv-VP2/gal was capable of infecting both HeLa and KG-1 cells, respectively. The inclusion of antibody against the whole AAV particle neutralized the infectivity of both wild-type rAAV/gal (Table 2) and rAAVsFv-VP2/gal on HeLa cells (Fig. 6E); however, it failed to neutralize the transduction of KG-1 cells by rAAVsFv-VP2/gal (Fig. 6F). This suggests that the anti-AAV antibody prevents both vectors from infecting HeLa cells by blocking the wild-type rAAV/receptor-binding step. rAAVsFv-VP2/gal transduction of KG-1 cells, however, was unaffected, presumably owing to infection mediated through binding of the fusion protein with CD34. When the rAAVsFv-VP2/gal was preincubated with antibody against either CD34 (my-10) or sFv, the infectivity of rAAVsFv-VP2/gal on KG-1 cells was blocked (Fig. 6G and 6H, respectively). This confirms that the interaction of sFv on the surface of rAAVsFv-VP2/gal with CD34 molecules on KG-1 cells is necessary for the transduction of KG-1 cells by rAAVsFv-VP2/gal, resulting in targeted gene delivery of chimeric rAAV vectors into $CD34^+$ KG-1 cells.

DISCUSSION

Efficient transduction of hematopoietic stem cells with gene delivery vectors is a long-standing objective of gene therapy, as these cells are progenitors for a number of cell types involved in various disease processes. Hence, after differentiation, stem cells can reconstitute the hematopoietic system and carry transgenes into desired lineages providing that the transgene exists as a stable component of the transduced cell. We chose AAV as a candidate targetable vector system primarily because these vectors have the capacity to transduce nondividing cells. It is assumed that the majority of stem cells would be in a quiescent state *in vivo*, hence, an injectable vector that is not dependent on cell division is essential.

Systemic *in vivo* delivery of viral vectors for gene therapy calls for vectors with appropriate specificity for the target tissue in question and minimal interaction with nontarget cells. Several groups have reported data demonstrating targeting of both retrovirus and adenovirus vectors to specific cell types *in vitro* (Somia *et al.*, 1995; Krasnykh *et al.*, 1996; Schwarzenberger *et al.*, 1996), however, no successful targeting of AAV vector has been reported. The difficulty, in part, has been due to limited knowledge of the mechanism of cell entry. Although a 150-kDa cellular membrane glycoprotein has been identified as a putative receptor for AAV (Mizukami *et al.*, 1996), the nature of the protein and its function in AAV infection, as well as virion protein(s) and corresponding domains involved in mediating receptor binding and virus entry, remain unknown.

We cloned a single-chain antibody against the human CD34 marker from the my-10 hybridoma. After expression in bacteria as a histidine-tagged protein, the purified CD34 sFv was similar to the my-10 MAb in its ability to block the binding of an MAb, ICH3, to CD34. Attempts to produce rAAV by entirely substituting sFv–AAV capsid fusion proteins for the corresponding wild-type protein failed to generate detectable intact particles with rAAV vector genome. A similar phenomenon was observed in the case of targetable retroviral vectors (Somia *et al.*, 1995). This suggests that complete replacement of one virion protein with sFv–VP fusion protein may cause a spatial hindrance for rAAV virion assembly or that the ratio of three virion proteins is not optimal for packaging. When only one sFv–AAV capsid fusion protein was included in the packaging reaction in the presence of all three wild-type proteins, intact rAAV chimeric vector particles containing sFv–Vp2 fusion protein were generated that significantly increased the transduction of KG-1 cells. The failure of rAAVsFv-VP1/gal and rAAVsFv-VP3/gal to transduce $CD34^+$ KG-1 cells may be the result of one of the following: (1) the sFv–VP1 or sFv–VP3 fusion proteins were not incorporated into the vector owing to conformational changes; (2) the sFv–VP1 and sFv–VP3 fusion proteins were presented in a conformation that does not allow for binding to CD34 molecules; (3) no internalization occurred for sFv–VP1 or sFv–VP3 fusion protein-encoding vectors; and (4) particles containing sFv–VP1 or sFv–VP3 fusion protein were not able to uncoat.

We examined in detail whether we have achieved targeted gene delivery with the rAAVsFv-VP2/gal vectors. Using both CsCl-purified and HPLC-purified rAAVsFv-VP2/gal vectors, we have observed expanded tropism of rAAV vectors to $CD34^+$ KG-1 cells, otherwise resistant to wild-type rAAV/gal transduction. The HPLC-purified rAAVsFv-VP2/gal has higher transduction efficiency on KG-1 cells than the CsCl-purified rAAVsFv-VP2/gal, relative to the titer on HeLa cells (Table 1). This may be due to either experimental variation or damaged infectivity of rAAVsFv-VP2/gal during CsCl purification. In the competitive binding assay, we observed the blocking of ICH3 antibody binding to CD34 molecules by particles of sFv–VP2 fusion protein containing rAAV, arguing that the improved transduction of KG-1 cells was mediated through the CD34 protein. Furthermore, the transduction of KG-1 cells by rAAVsFv-VP2/gal was inhibited when the modified rAAV virions were preincubated with antibody against CD34 and sFv. This not only confirmed the specificity of transduction via CD34 molecules, but also suggested that there were sFv–VP2 fusion proteins on the surface of the vector.

We observed low or no transduction of wild-type rAAV/gal vectors on KG-1 cells. This serves as a good control to rule out the possibility of "pseudotransduction," as observed by several researchers with crude preparation of rAAV vectors. It is not clear whether the KG-1 cells had low or no expression of the AAV receptor, and thereby were resistant to rAAV infection. Since vector containing the vSFv-VP2 fusion protein yielded reasonable levels of gene transduction in these cells, it is possible that the CD34 molecules mediated rAAV entry via a different mechanism from the AAV receptor. This illustrates that targetable rAAV vectors can efficiently deliver transgenes via receptors that are considerably different from those of the wild-type route of entry.

Although further improvements may be needed for this modified rAAV vector to be practically useful for *in vivo* gene therapy applications, we consider that the increased gene delivery to KG-1 cells by more than 100-fold to be highly significant, particularly in the case of purified rAAV vectors (Tables 1 and 2). Moreover, in this pilot study the CD34 marker protein was chosen as the target molecule for the test of principle and it may not be the ideal receptor for viral entry. Subsequent studies will determine the generality of modifications to VP2 as a

means of targeting AAV vectors via specific cell surface proteins. As indicated above, a useful targetable vector should have reduced binding capability to its native receptor. This was not the goal in these first-generation targetable AAV vectors, as all three wild-type capsid proteins are present. Currently, we are attempting to mutagenize the AAV capsid so that native binding is significantly reduced or eliminated. Nevertheless, this study represents the first positive step toward the development of a targeted rAAV vector.

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